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Applicant:	FODSTAD et al.	Examiner:	Sisson, B.
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Title:	IMMUNO-MAGNETIC CELL SEPARATION USED IN IDENTIFICATION OF GENES ASSOCIATED		

DECLARATION UNDER 35 U.S.C. §1.132

I, Øystein Fodstad, M.D., Ph.D. am an inventor of the above-referenced application. I am head of the Department of Tumor Biology and Director, Institute for Cancer Research Council, at The Norwegian Radium Hospital, University of Oslo and have extensive experience in the biotechnical arts, including antibody production and separation. I have read and understood the Official Actions dated July 31, 2001 and February 27, 2002 issued by Examiner Sisson.

My invention is a method for identifying genes differentially expressed between cells isolated from different tissues, the method comprising:

(A) detecting target cells from a first and a second tissue;

(B) obtaining nearly 100% specific target cells by repeatedly immunomagnetically isolating, *in vitro*, said first and second tissue target cells;

(C) determining levels of mRNA expression within said first and second tissue target cells;

(D) comparing the levels of mRNA expression in said first and second tissue target cells; and

(E) based upon the comparison in step D, identifying the genes differentially expressed between said first and second tissue target cells, wherein at least one of said first and second tissue target cells are tumor cells, in order to recognize previously unknown genes possibly involved in determining metastatic characteristics of cancer cells.

One advantage of my invention is that it allows for easier identification of genes differentially expressed between primary tumors and metastatic tumors. Identification and study of such genes could prove invaluable in determining, for example, the mechanisms associated with metastasis. Prior to my invention, it was not thought possible to perform meaningful gene closing experiments on specimens of solid tumors and metastases for the purposes of identifying genes with site-specific expression. This is because it was thought impossible to separate tumor cells from normal cells without first culturing the cells and performing manipulations to get rid of the normal cells. However, the culture conditions resulted in changes in gene expression relative to *in vivo* expression. These changes in gene expression rendered gene cloning experiments for the identification of genes involved in the metastatic process meaningless when culturing was performed to isolate tumor cells from surrounding normal cells. Yet without the isolation of tumor cells from surrounding normal cells, the background gene expression of the normal cells posed similar problems. My invention overcomes these problems by providing a method by which target cells can be separated form a cell population in order to identify the gene sequences from the target cells in a specific cell population environment.

At the time of my invention, a scientist in the field of molecular and cellular biology would have known how to perform the following method steps individually:

1. Immunomagnetically isolate, in vitro, cells obtained in vivo;
2. Determine levels of mRNA in cells; and
3. Compare levels of mRNA between cells and to identify whether the mRNA is differentially expressed.

IMMUNOMAGNETIC ISOLATION, *IN VITRO*, OF CELLS OBTAINED *IN VIVO*

At the time of my invention, techniques for immunomagnetically isolating cells were known. For example, page 4 of the application refers to two PCT patent applications, WO94/07139 and WO95/24648, which teach the ability to (A) detect target cells from a tissue and (B) obtain nearly 100% specific target cells by repeatedly immunomagnetically isolating, *in vitro*, the target cells.

Briefly, these results can be achieved by a method which involves binding of monoclonal antibodies that specifically recognize antigens present on tumor cells, and not on the normal cells

in question, or for other purposes to specified subpopulations of normal cells, to magnetic particles, either directly or to beads first covered with antibodies specifically recognizing the immunologic isotype of the respective antibodies that bind to the tumor cells. Such antibodies may be of the IgG type or may be a fragment of IgG or IgM. The targeting antibodies may be attached directly to the magnetic particles, or the binding to cells can take place by using beads coated with antibodies specifically recognizing the Fc portion of the said individual antibodies already bound to the target cells. The antibody coated magnetic beads are mixed with the suspension of cells to be examined, incubated for 30 minutes at 0-10°C, preferably 4 °C under gentle rotation. Samples of the cell suspension are then transferred to a cell counting chamber, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The visualization of target cell-particle complexes, the rosettes, makes it simple to directly spot the target cells with up to 100% specificity.

In WO95/24648, the above described method is improved by further transferring the suspension of target cell-particle complexes to a cell filtering device, and after performing the filtration the target cell-particle rosettes can be viewed microscopically on the filter membrane or grown in a physiological base culture medium, e.g. without separation the target cells from the particles, to be characterized for the presence of specific biochemical and biological features.

Wang, et al. (Pathology Oncology Research 1995) teach an immunomagnetic procedure for isolating specific cells from a mixed cell population using antibody-coated beads. Thus, at the time the invention was made, one of ordinary skill in the art would know how to isolate cells using antibody-coated beads.

DETERMINATION OF LEVELS OF MRNA WITHIN A TISSUE

At the time of my invention, one would have known how to determine levels of RNA within a tissue. Several well known techniques, such as reverse transcriptase PCR (RT PCR), in situ hybridization, RNAse protection assays, and Northern blots are available for such purposes. A search of the PubMed Database at http://www.ncbi.nlm.nih.gov/PubMedOld/medline.html for journal articles published in the year 1995 describing RT PCR resulted in 1,315 hits. Similar searches for publications in 1995 were performed for in situ hybridization, RNAse protection, and Northern blot. The results are presented in table 1.

Table 1. Number of publications in PubMed database disclosing methods and techniques associated determinations of RNA levels

Search Term	Hits for year 1995
RT PCR	1,315
In Situ Hybridization	4,878
RNAse Protection	395
Northern Blot	2,007

The number of hits obtained in Table 1 should be considered as a floor for the actual number of journal articles disclosing these techniques as the search terms entered do not cover all possible deviations in nomenclature. Regardless of inconsistency in nomenclature, more than 7,000 publications disclosed well-known techniques for determining RNA levels in tissues in 1995 alone.

Determining the level of RNA in a tissue was well within the ability of the skilled artisan at the time of filing of the above-referenced application. However, a brief general overview of RT PCR and Northern blot techniques may provide useful for discussion of the appended references.

In an RT PCR assay, cDNA is generated by incubating RNA, which has been isolated by known techniques, with reverse transcriptase enzyme. The cDNA can then be amplified by PCR using oligonucleotide primers that hybridize to an upstream and a downstream portion of a cDNA molecule of interest (which corresponds to the RNA species of interest). PCR amplifies, or produces, in an exponential fashion, copies of the cDNA molecule of interest between the areas in which the primers hybridize. The amplified DNA can be quantified in a visual or by an automated process. A "housekeeping" gene can be amplified to correct for unequal amounts of starting material. A housekeeping gene is a gene that is generally present at similar levels in all cells. An example of a housekeeping gene is β-actin. This correction is typically achieved by dividing the amount of signal generated from the amplified region of interest by the signal generated from the amplified portion of the housekeeping gene.

In Northern blot analysis, RNA is isolated from a tissue by known techniques, separated by apparent molecular weight by applying a voltage differential across a gel into which the RNA is loaded, and either electrically or osmotically transferred to a membrane, such a polyvinyl fluoride membrane. The membrane is then typically probed for the presence of an RNA species with an excess of a cDNA molecule capable of hybridizing to the RNA species of interest. The membrane is washed to remove unhybridized cDNA molecule. Attached to the cDNA molecule is a detectable label that can be exploited to quantify the number of RNA molecules of the species of interest to which the labeled cDNA molecule bound. Sometimes an RNA molecule, rather than a cDNA molecule is used as a probe. Additionally, a similar technique known as the dot blot can be used. In a dot blot assay, the RNA containing the species of interest is directly blotted onto the membrane.

In Northern blot analysis it is often desired to probe for a "housekeeping" RNA species in addition to the species of interest. A "housekeeping" RNA species is an RNA species that is generally present at similar levels in all cells. An example of a "housekeeping" RNA species is 18S rRNA. Probing for a housekeeping RNA species in addition to the RNA species of interest allows for corrections for uneven amounts of RNA loaded in various lanes of the gel. This correction is typically achieved by dividing the amount of signal generated from the probe to the RNA species of interest by the signal generated from the probe for the housekeeping RNA species. The corrected signal can be quantified visually or through an automated process.

As examples of specific references that disclose techniques for determining RNA levels in a tissue, please find enclosed Appendices 3 -6. Appendix 3 is Mælandsmo et al., Cyclin kinase inhibitor $p21^{WAF1/CIP1}$ in malignant melanoma, *American Journal of Pathology*, 149(6):1813-1822 (1996). This journal article discloses Northern blot analysis for detecting *WAF1/CIP1* RNA levels in malignant melanomas. Specifically, a *WAFI/CIP1* cDNA probe was used to probe total cellular RNA, with an 18S rRNA probe being used to correct for unequal loading. Appendix 4 is Mælandsmo et al., Reversal of the *in vivo* metastatic phenotype of human tumor cells by an anti-*CAPL* (*mts1*) ribozyme, *Cancer Research*, 56: 5490-5498 (1996). This journal article discloses the use or Northern blot analysis and RT-PCR to quantify the level of *CAPL* RNA. In the Northern blot analysis 18S RNA was used to correct for unequal loading after scanning an audioradiogram in a densitometer. In the RT PCR assay, β-actin was used to correct for differences in starting material. The quantification was based on a colorimetric assay.

Appendix 6 is Deggerdal et al., Semiquantitative polymerase chain reaction for t(14;18) in follicular lymphomas: A colorimetric approach., *Laboratory Investigation*, 72(4): 411- 418 (1995). This journal article disclosed a method for quantifying translocation-positive cells. While this reference does not teach the quantification of RNA from a tissue, one skilled in the art would recognize that the methods disclosed in this reference would be applicable to quantification of RNA by the additional initial step generating cDNA by using reverse transcriptase.

COMPARISON OF MRNA LEVELS BETWEEN TWO DIFFERENT TISSUES TO DETERMINE WHETHER MRNA IS DIFFERENTIALLY EXPRESSED

At the time of my invention, one would have known how to compare levels of RNA between two different tissues to determine whether mRNA was differentially expressed. Several well known techniques, such as differential display and subtractive hybridization analyses were available for such purposes. A search of the PubMed Database at http://www.ncbi.nlm.nih.gov/PubMedOld/medline.html for journal articles published in the year 1995 describing differential display analysis resulted in 213 hits. A similar search for publications in 1995 that described subtractive hybridization analysis resulted in 54 hits. A brief general overview of these techniques is given.

In a differential display assay, the general strategy is to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and PCR. These short sequences are then displayed on a sequencing gel for determination of differential expression. Pairs of primers are selected so that each will amplify DNA from about 50 to 100 mRNAs because this number is optimal for displaying on the gel. Selection of 3' primers takes advantage of the polyadenylate [poly(A)] tail present in most eukaryotic mRNAs to anchor the primer at the 3' end of the mRNA. The methodology can be modified to utilize biotinylated anchored primers (5'-T_{11}VN-3') coupled to streptavidin-coated magnetic particles for mRNA extraction, as well as the subsequent first strand cDNA synthesis and cDNA amplification. The cDNA is amplified using the anchored primer in conjunction with an upstream arbitrary primer (AP), and using [^{35}S]dATPα as label. The AP is a 5' primer of arbitrary base sequence, annealing to the cDNA at randomly distributed positions relative to the poly(A) tail. To obtain this the PCR reaction is performed with a low annealing temperature. The PCR products that are generated in this

reaction are resolved on a standard denaturing sequencing gel, which is subsequently dried and subjected to autoradiography. PCR fragments unique for the tissue of interest are identified and excised from the gels, and the eluted products are reamplified using the original set of primers and the same thermal cycling condition. The reamplified products are then sequenced for identification and used as probes to verify differential expression by Northern blot hybridization. The reproducibility of each step in this protocol has been documented.

The basic methodology of subtractive hybridization analysis is also designed to select for genes expressed uniquely or preferentially in one of a pair of closely related cell populations. cDNA-RNA hybridization distinguishes mRNAs that are equally expressed in both cell populations from those unpaired mRNAs that are unequally expressed in the cell population of interest. Again, poly(A)$^+$RNA (i.e., mRNA) is purified and cDNA subsequently synthesized with an isotope label from the cell population of interest. The prelabeled single-stranded cDNA is then hybridized with mRNA from the referenced cell population. The hybridization reaction mixture is loaded onto a separating column, such as a hydroxylapatite column, to segregate double-stranded hybrids from single-stranded cDNA. The effluent is often rehybridized to mRNA from the reference cell population (second subtraction) to collect the second effluent of cDNA from the cell population of interest. The eluted single-stranded products are subsequently hybridized to a cDNA library, preferably two or more times to exclude false positives, and the final positive clones are then isolated and amplified by PCR using the linker sequences of the cloning vector as primers. The resulting PCR products are then sequenced for identification and used as probes to verify differential expression by Northern blot hybridization. Several modifications in the subtractive hybridization methodology have been reported to be applied to a wide range of biological problems.

Clearly, these techniques were known and available to those interested in determining whether mRNA is differentially expressed between two tissues.

Representative references teaching such techniques include:

1. Ebralidze et al., *Genes Dev.* **3**: 1086-1093 (1989)
2. Liang and Pardee, Science 257:967-971 (1992)
3. Liang et al. CAncer Res. 52:6966-6968 (1992)
4. Rosol et al., Biotechniques 21:114-121 (1996)
5. Sobel, J. Natl. CAncer Inst. 82:267-276 (1990)

6. Lee et al., PNAS 88:2825-2829 (1991)
7. Sager, Curr. Opin. Cell Biol, 4:155-160 (1992)
8. Owens and Cohen, Cancer Metastasis Rev. 11:149-156 (1992)

EVIDENCE THAT TEACHINGS OF APPLICATION CAN BE APPLIED TO ACHIEVE MY INVENTION

My invention has been successfully used to isolate nucleic acid sequences potentially involved in metastasis. Three scientific journal articles showing successful use of my invention are attached to this declaration. First, Ree et al., "Expression of a novel factor in human breast cancer cells with metastatic potential," *Cancer Research* **59**: 4675-4680 (1999), shows the isolation of a nucleic acid sequences that may play a role in metastasis. Human breast carcinoma cells were injected into the systemic circulation of immunodeficient rats. Resulting CNS metastatic tumors were isolated from the rats. Metastatic cells were isolated using immunomagnetic beads coated with an antibody that was reactive with human cells. The segregated cell population was compared with the injected cells by means of differential display analysis. Two nucleic acid molecules that may play a role in metastasis were identified. The biological mechanism of one of these nucleic acid molecules, com1, was studied in a second journal article (Bratland et al., Expression of a novel factor, com1, is regulated by 1,25-dihydroxyvitamin D3 in breast cancer cells, *Cancer Research* **60**: 5578-5583, 2000) revealing potential insights into the metastatic process. In a third journal article (Ree et al., "Differential display analysis of breast carcinoma cells enriched by immunomagnetic target cell selection - gene expression profiles in bone marrow target cells", *Int. J. Cancer*, **97**:28-33, 2002) additional candidate nucleic acid molecules were identified.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such false statements may jeopardize the validity of the application or any patent issued thereon.

Date:_____

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Øystein Fodstad

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ARTICLE

An Effective, Direct Immunomagnetic Procedure for Purging Acute Lymphoblastic Leukemia Cells from Human Bone Marrow*

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The AB4 monoclonal antibody, which recognizes an HLA-DR epitope, was found to bind to a high percentage of malignant blast cells in samples obtained from 27 patients with ALL. These included 11 of 11 cases with c-ALL, 3 of 7 with pre-pre-B, and 8 of 9 cases with pre-B ALL. AB4 was used together with anti CD10 and anti CD19 antibodies and super-paramagnetic particles for developing a direct immunomagnetic procedure for purging human bone marrow of leukemic cells. In model experiments with KM3 cells admixed to mononuclear bone marrow cells, the individual antibodies each removed 2.8–3.1 logs and 3.6–4.1 logs of tumor cells with one and two purging cycles, respectively. In comparison, the efficacy of a mixture of the three antibodies was 4.4 logs with one treatment cycle, and > 5 logs with repeated treatments. Whereas the use of a commercially available anti-HLA-DR antibody resulted in a 90% reduction in the survival of CFU-GMs and normal blast colonies, AB4 had only a moderate effect on the progenitor cells (46% and 30% reduction). In conjunction with autologous transplantation, bone marrow from a patient was purged with the antibody mixture and 50% of the CFU-GMs and 47% of the $CD34^+$ cells remained after treatment. The patient showed a normal engraftment, reaching a level of 0.5×10^9/l neutrophils by day 20 and 20×10^9/l platelets by day 30. It is concluded that the antibody cocktail may safely and effectively be used for rapid autograft purging in patients with c-ALL, and also in phenotypically selected cases with other subtypes of ALL. (Pathology Oncology Research Vol 1, No1, 32–37, 1995)

Key words: bone marrow, leukemia, transplantation, purging

Introduction

Autologous bone marrow transplantation (ABMT) is considered as an alternative therapy for patients with high risk acute lymphatic leukemia (ALL) who lack a histocompatible allogeneic donor.[1,2] In leukemia, the possible contribution of bone marrow (BM) purging to the efficacy of ABMT is unknown, as prospective clinical studies have not been performed.[1,2] However, gene-marking studies of autografted cells to trace the origin of relapse after ABMT have indicated that tumor cells remaining in the reinfused marrow contribute to recurrence of the disease.[3] The possible advantage of BM purging is further supported by results in patients with follicular lymphomas which indicate that efficient purging improves disease-free survival.[4]

In ALL patients the malignant event may occur in an early pluripotent stem cell. Hence, for purging purposes anti-HLA-DR antibodies that bind strongly to B-cell precursor ALL cells might be candidates for inclusion in the panel of antibodies used.[5,6,7] However, since HLA-DR gene products are also expressed on cells of the pluripotent stem cell compartment, it has been anticipated that purging with antibodies recognizing these proteins may also deplete early progenitor cells from the graft. We have, however, previously shown in a patient with non-Hodgkin lymphoma that a BM

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autograft purged with our AB4 anti-HLA-DR antibody[8] resulted in rapid and sustained engraftment.[9] Thus, it seems likely that the corresponding antigen, encoded by the B3 gene of the DR region, is not expressed in the most immature hematopoietic progenitor cells. To study whether the AB4 monoclonal antibody (MAb) binds to B-cell precursor ALL cells we first performed immunophenotyping of leukemic blasts in samples from patients with such disease, and found that the AB4 antigen was expressed on B-cell precursor ALL blasts in 22 of 27 patient samples.

When purging BM with immunobeads, the relevant antibodies can be used either in a direct or an indirect procedure.[10,11,12] With the direct method, the primary antibody is in advance attached to the beads, thereby eliminating both one incubation and a washing step.[10] This approach was tested in model purging experiments with a mixture of AB4, anti CD10 and anti CD19 antibodies, and highly promising results were obtained.

Materials and Methods

MAbs and immunobeads

An anti HLA-DR (IgG_{2A}) antibody was obtained from Becton Dickinson (Mountain View, CA). Our AB4 (IgM) antibody binds to an antigen encoded by the B3 locus of the DR gene. The F103.11 anti CD10 antibody (IgG_1) was a gift from T. Plesner (Amtssygehuset, Herlev, Copenhagen, Denmark), and the HD37 anti CD19 MAb (IgG_1) was kindly provided by B. Dörken (Robert Rossler Klinik, Free University, Berlin, Germany). The antibody against terminal deoxynucleotidyl transferase (TdT) was obtained from DAKO (Santa Barbara, CA). Dynabeads M-450 directly coated with the antibodies were delivered by Dynal (Oslo, Norway).

Immunophenotyping

Fresh BM or peripheral blood samples from 27 ALL patients, containing 80% blasts or more, were isolated by standard Ficoll-Hypaque density gradient centrifugation, and cell surface or intracytoplasmic antigens were examined by indirect immunofluorescence. MAbs to lymphoid-, myeloid-specific and non-lineage associated antigens, including the anti-HLA-DR and AB4 antibodies, were used as previously described.[13] Positive reaction is defined as $\geq$ 20% of the blasts expressing the surface markers or $\geq$ 10% blasts containing intranuclear/cytoplasmatic markers.[14] Immunophenotypic classification of B-cell precursor (i.e. pre-pre-B, common, Pre-B ALL) subtypes followed criteria described elsewhere.[5]

Tumor and BM cells

The KM3 non-T ALL cell line was cultured at 37°C in RPMI 1640 medium (RPMI) containing 10% fetal calf serum (FCS). Cultures were passaged frequently to assure that cells used in the experiments were in a proliferative log phase.

Normal BM cells were obtained from patients with non-hematological cancers aspirated for diagnostic purposes at the Norwegian Radium Hospital. None of the samples showed BM involvement of the disease as judged by BM biopsies and smears. Mononuclear cells were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and washed twice in phosphate-buffered saline (PBS) before use.

Assays of tumor cells and BM progenitor cells

The number of residual clonogenic KM3 tumor cells in control and purged samples was determined in a soft agar assay as previously described.[8] After 14 days of incubation at 37°C, colonies of more than 50 cells were counted using a Zeiss stereo microscope. The plating efficiency (PE) was defined as the number of colonies formed, expressed as the percentage of the number of viable cells plated. With the concentration of BM cells used, no colonies were formed in the absence of tumor cells.

The clonogenic capacity of the normal BM progenitor cells after purging was assessed in the CFU-GEMM and CFU-GM assays.[8] The blast colony formation assay on irradiated stromal feeder layers was performed as previously described.[15,16]

Incubation conditions and cell separation

10^6 KM3 cells mixed with $9x10^6$ mononuclear BM cells per ml in RPMI containing 10% FCS were incubated in 10 ml plastic tubes with the desired number of AB4, anti CD10 and anti CD19 immunobeads at 4°C for 30 min with gentle rotation. The concentration of beads was chosen on the basis of the total number of antibody-binding B-cells. The optimal ratio of beads to total nucleated cells was found to be 2 to 1 (data not shown), and this ratio was used in all experiments. The effect of the anti HLA-DR monoclonal antibody was tested by the indirect method. 10^7 mononuclear BM cells per ml of RPMI containing 10% FCS were incubated in 10 ml plastic tubes for 30 min at 4°C with 10 mg/ml of the anti HLA-DR antibody. The cell suspension was washed twice with cold PBS, resuspended to a final concentration of $1x10^7$ cells/ml in PBS and incubated with the desired number of Dynabeads SAM ST at 4°C for 30 min with gentle rotation.

In all cases the resulting cell/bead aggregates were removed by placing a Cobalt-Samarium magnet to the wall of tubes. After 1 min, the suspension was aspirated, and when desired a new batch of immunobeads was added to the cell suspension and the cycle was repeated.

A patient in first relapse of unclassified high grade non Hodgkin lymphoma, who had advanced disease with the BM heavily infiltrated with lymphoblasts expressing CD10,

CD19 and the AB-4 epitope, was brought into complete remission with second line chemotherapy. Bone marrow was harvested and the mononuclear cells were isolated using a CS-3000 Plus Blood Cell Separator (Baxter Healthcare Corporation, Deerfield, IL). The mononuclear cell fraction was purged with the AB4, anti CD10 and anti CD19 immunobeads under similar conditions to those described above, employing a MaxSep Magnetic Cell Separator (Baxter Healthcare Corporation). The number of CFU-GMs and CD34$^+$ cells before and after purging was estimated. Twenty days later, the patient was treated with total body irradiation in doses of 1.3 Gy twice daily for five days and with 60 mg/kg body weight of cyclophosphamide daily for two days. Two days after finishing the high dose treatment the purged BM was reinfused into the patient.

Results

Immunophenotype of blast cells in BM and peripheral blood of ALL patients

BM or peripheral blood cells from 27 patients with diagnosed ALL were immunophenotyped with the different MAbs. Seven patients were found to have pre-pre-B-cell, 11 c-ALL and 9 pre-B-cell disease, according to criteria described by Does-van den Berg et al.[5] The expression in each of these subclasses of the antigens recognized by anti-TdT, anti-HLA-DR, and AB4 is listed in *Table 1*. It can be seen that all c-ALL patients, 3 of the 7 pre-pre-B ALL, 8 of 9 pre-B ALL expressed the AB4-associated antigen. The expression was heterogenous, ranging from 25% to 95% positive cells. When comparing the immunoreactivity of AB4 to that of anti-TdT, which is known to bind to malignant blasts but reacts with only about 1% of normal blast cells,[14] it can be concluded that AB4 binds a high fraction of leukemic c-ALL cells. Moreover, also in most of the AB4 positive pre- and pre-pre-B ALL cases the percentage of immunoreactive cells was high. The fraction of AB4 positive cells was consistently lower than that seen with the other HLA-DR antibody, which often bound to the surface of a higher percentage of cells than those stained intracytoplasmatically with the anti-TdT antibody.

Importantly, all c-ALL tumor cells expressed antigens recognized by at least one of the anti CD19, anti CD10, and AB4 MAbs (not shown), indicating that a mixture of these antibodies should be well suited for BM purging with immunobeads in this subgroup of ALL patients.

Recovery of progenitor cells after immunomagnetic purging with HLA-DR antibodies

In preclinical studies, HLA-DR antibodies used for BM purging have been reported to deplete hematopoietic stem cells. To further clarify the hematopoietic toxicity of immunomagnetic purging with AB4 beads, a comparison between the use of AB4 and the more broadly reactive HLA-DR antibody with immunobeads was performed. It is shown in *Table 2* that after 2 purification cycles with AB4, 54% of CFU-GM and 70% of blast colonies survived, whereas the use of the other HLA-DR antibody resulted in both assays in only 10% recovery of the normal progenitor cells.

Table 1. **Fraction of anti-TdT, AB4, and anti-HLA-DR reactive blast cells in bone marrow or peripheral blood from patients with B cell precursor ALL**

Immunophenotypic subclass	*Patient no*	*Marker expression on B-cell precursor precursor ALL blasts (%)**		
		TdT	*AB4*	*HLA-DR*
Pre-pre-B-ALL (TdT$^+$, CD19$^+$)	1	80	41	91
	2	85	49	82
	3	70	69	73
	4	65	11	82
	5	74	7	90
	6	50	0	44
	7	95	5	91
c-ALL (TdT$^+$, CD19$^+$, CD10$^+$, cyIgM$^-$)	1	62	52	67
	2	80	74	97
	3	90	52	94
	4	90	53	83
	5	31	61	78
	6	90	77	79
	7	75	70	91
	8	75	78	93
	9	90	50	91
	10	95	25	94
	11	95	47	96
Pre-B-ALL (TdT$^+$, CD19$^+$, CD10$^{-/+}$, cyIgM$^+$, sIgM$^-$)	1	40	75	n.d.
	2	90	28	–
	3	60	0	–
	4	90	39	–
	5	90	95	–
	6	90	90	–
	7	60	58	–
	8	85	85	–
	9	68	63	–

* All results given as percent positive cells. Positive reaction was defined as 20% of the blast cells expressing the surface (AB4, HLA-DR) markers, or 10% of the blasts containing the cytoplasmic (TdT) marker.

Efficacy of tumor cell depletion

We compared the efficacy of KM3 tumor cell removal by using beads directly coated with the primary MAbs. It is shown in *Fig.1* that with individual antibodies and one cycle of purification, a tumor cell depletion 2.8–3.1 logs was obtained. A second cycle of purification increased these

numbers to 3.6-4.1 log cell removal. When a mixture of all three antibodies was employed, 4.4 logs of tumor cells were removed with one cycle and no colony formation was observed after a second purging cycle. The latter result represents a tumor cell removal efficacy of at least 5 logs. Altogether, the data suggest that there is no complete overlap in tumor cell expression of the three antigens.

***Table 2.* Recovery of blast colonies and progenitor cells after two cycles of immunomagnetic purging using either the anti-HLA-DR antibody and SAM-beads or the AB4-beads**

	*No of colonies formed**	
	CFU-GM/$2x10^5$ MNC	Blast colonies $2x10^5$ MNC
Untreated control	160 ± 26 (100%)	189 ± 33 (100%)
HLA-DR/SAM-IB	15 ± 14 (10%)	19 ± 14 (10%)
AB4-IB	87 ± 32 (54%)	148 ± 38 (70%)

Fresh bone marrow cells ($1x10^7$/ml) were incubated in RPMI 1640 medium with the HLA-DR Mab followed by M-450 SAM beads, or with the direct AB4 beads. Conditions as in *Fig 1.*

* The results represent the mean SD of three independent experiments, each in triplicate, and the numbers in parentheses show the number of colonies relative to that in the untreated control.

Effect of immunomagnetic purging on hematopoietic stem cell recovery in a patient

The mixture of directly coated AB4, anti CD10, and anti CD19 immunobeads had, in an experimental setting, given a moderate effect on BM progenitor cells, with a 50% recovery of CFU-GMs (data not shown). When a full scale immunomagnetic purging procedure, employing the same immunobeads and with the same ratio of target cells and beads, was used on BM harvested from a patient with a relapsed high grade lymphoma, the recovery of CFU-GM cells and $CD34^+$ cells was 69% and 48%, respectively. After high dose treatment of the patient with total body irradiation and chemotherapy, the BM was reinfused. The patient showed a normal reconstitution with a level of 0.5×10^9 /l neutrophils reached on day 20, and of 20×10^9 platelets on day 30.

***Table 3.* Recovery of mononuclear BM cells, progenitor cells and $CD34^+$ cells after 2 cycles of immunomagnetic purging using a cocktail of AB4, anti-CD10 and anti-CD19 immunobeads**

Total no of	*Before purging*	*After purging*	*% Recovery*
MNC cells	4.5×10^9	2.3×10^9	51
CFU-GM cells	6.5×10^9	4.5×10^9	69
$CD34^+$ cells	1.7×10^8	0.8×10^8	47

Mononuclear bone marrow cells were isolated by using the CS3000 cell separator, and purging with immunobeads was performed with the MAX SEP cell separator as described in Materials and Methods.

Discussion

HLA-DR antigens are known to be strongly expressed on the surface of B-cell precursor ALL cells, also in the most immature types of the disease. Therefore, if the toxicity to normal BM progenitor cells was acceptable, the inclusion of an anti HLA-DR antibody in a panel of MAbs used for immunobead purging would be expected to be advantageous. Initial experiments showed that AB4 anti HLA-DR immunobeads were quite efficient in removing leukemic cells admixed to BM. Moreover, a good recovery of normal progenitor cells was seen after purging with these beads. Hence, it was of interest to examine the binding profile of AB4 in blast cells from ALL patients to examine the possibility of using this antibody for BM purging in such disease. Immunophenotyping of BM and peripheral blood cells from 27 patients showed that 11 of 11 c-ALL, 3 of 7 pre-pre-B ALL, and 8 of 9 pre-B ALL patients expressed the antigen recognized by AB4. The AB4 antigen was not uniformly expressed on the ALL blasts, but if used in a mixture with anti CD10 and anti CD19 antibodies the heterogeneity in antigen expression on B-cell precursor ALL cells should be well covered.

In model experiments, the mixture of the 3 immunobeads removed more than 4 log KM3 tumor cells with one, and at least 5 logs with two purging cycles. Importantly, in these experiments a procedure with the specific antibodies attached directly onto magnetic beads was used, thereby eliminating one incubation and one washing step compared to the more commonly used indirect immunobead method. With the purging efficacy obtained, the direct method offers clear advantages with respect to speed and simplicity, and two cycles of purification can be performed within one hour.

The present, and also previous results,[8,9] showed a good recovery of hematopoietic progenitor cells after immunomagnetic purging, both with AB4 beads alone and when used in the mixture. Therefore, it can be concluded that AB4 recognizes an epitope that is not expressed, or has a low level of expression, in hematopoietic stem cells. In contrast, purging with the other anti-HLA-DR antibody resulted in depletion of 90% of CFU-GMs and blast colonies *(Fig.1).*

Philadelphia chromosome positive (Ph^+) ALL is associated with poor prognosis and such patients may be considered for high dose treatment at first complete remission.[18] It has been reported that 55% of adult c-ALL patients have the typical BCR/ABL rearrangement and that its presence correlates with poor overall survival and remission duration.[17] In our study, all the 11 phenotyped c-ALL patients had Ph^+ blast cells, which also expressed the antigens recognized by the anti CD10, CD19 and AB4 antibodies. Based on these findings a phase-I study in Ph^+ ALL patients treated with ABMT and BM purged with the directly coated beads has been initiated. In such patients

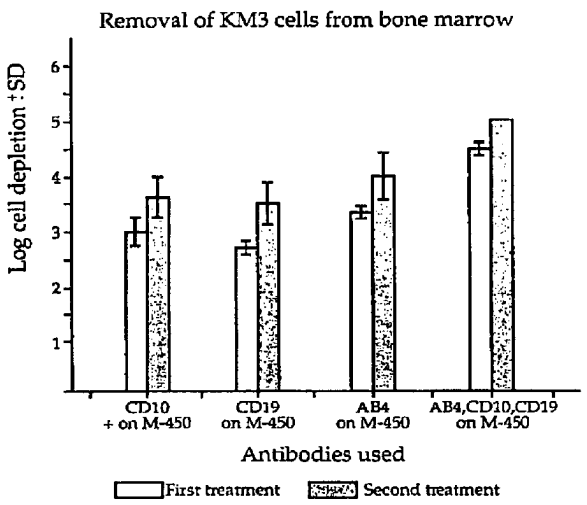

Figure 1. Efficacy of immunobeads involving three different antibodies in removing KM3 leukaemia cells. One x10^6 KM3 cells admixed with 9x10^6 MNC bone marrow cells were incubated with M-450 beads coated individually with different primary antibodies. The bead/total nucleated cell ratio was 2 to 1. After magnetic separation the number of remaining tumor cells was determined as described in "Material and Methods". The results represent the mean ± SD of 3 independent experiments in triplicate.

the purging efficacy may be assessed by a polymerase chain reaction (PCR) method that detects the presence of possible BCR-ABL chimeric transcripts remaining in the autograft. The procedure is in principle similar to that recently reported for use in patients with follicular non-Hodgkin's lymphoma.[19] It should be noted that the immunomagnetic method facilitates the use of PCR procedures for analyzing the purging efficacy. Thus, since the target cells are removed from the autograft, problems with false positive results are avoided. In contrast, when methods involving complement or immunotoxins are used positive PCR amplification may result from contaminating target DNA carrying breakpoint sequences liberated from lysed tumor cells. Moreover, with immunobeads the risk of false negative results is also greatly diminished, as PCR amplification of specific sequences can be performed on the cell suspension containing the bead-removed target cells.

The insignificant effect of AB4 immunobeads on normal stem cells has been shown in a clinical study in which 4 non-Hodgkin lymphoma patients autotransplanted with BM purged with CD19 and AB4 beads showed normal and sustained engraftment.[20] These results were confirmed here in a patient autotransplanted with BM purged with AB4, anti-CD10 and anti-CD19 immunobeads. It is concluded that the direct immunobead technique with these antibodies can safely and efficiently be used to eradicate ALL cells in BM autotransplants.

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